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13. ABSTRACT (Maximum 200 Words)

Telomeres are the protective ends of chromosomes that are necessary for maintaining the viability of a cell and the integrity of its genome. One essential function of telomeres is to protect the chromosome end from being degraded by nucleases that are normally present in any cell. Unprotected chromosome ends could fuse to each other leading to loss of genetic material, genomic instability, and subsequent carcinogenesis. The focus of my research is two-fold: (1) to understand the mechanism of chromosome end protection in the well-studied model organism, *S. cerevisiae;* and (2) to extend the knowledge gained in yeast to humans by identifying human homologues of the essential proteins involved in end protection. Experiments geared toward addressing the first goal have led to the identification of two residues in Cdc13 that are phosphorylated *in vivo*. Phosphorylation of these two residues is likely to be important in directing the overall low level of telomerase in to short telomeres that require elongation. In order to extend these and any future observations to humans, human homologues of Cdc13, Stn1 and Ten1 must first be identified. Increasing data suggests that Pot1 is the human homologue of Cdc13. We have reported the identification of a putative human Stn1 we are currently characterizing it as well.

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Introduction

Telomeres are the protective ends of chromosomes that are necessary for maintaining the viability of a cell and the integrity of its genome. Due to the inability of semi-conservative replication to replicate to the very end of the chromosome, telomeres shorten with every cell division and eventually diminish. This eventually leads to cellular senescence or apoptosis[16]. In order to repair these telomere ends, cells can use telomerase to elongate these protective ends. Normal breast tissue does not express telomerase and therefore has a finite lifespan while breast cancer cells with aberrant telomerase expression are able to bypass this limit and proliferate continuously. Telomeres must also protect the chromosome end from being degraded by nucleases that are normally present in any cell. Unprotected chromosome ends could fuse to each other leading to loss of genetic material and genomic instability. It has recently been demonstrated that such telomere dysfunction and subsequent bridge-fusion-breakage cycles may drive the chromosomal instability associated with the benign-to-malignant transition in breast cancer[1].

Cdc13, Stn1 and Ten1, have been shown to be critical regulators of telomere function in the budding yeast, *S. cerevisiae* [5-7, 14]. Recent data in the lab that is not discussed here suggests that these proteins form a heterotrimeric complex in a manner that is analogous to another protein essential to eukaryotic DNA replication - replication protein A (RPA)[20]. These observations have led us to propose that Cdc13, Stn1 and Ten1 may form a telomere-specific RPA that we refer to as the CST complex. Like the canonical RPA, we propose that the CST complex can mediate replication through the enzyme telomerase and associates with DNA repair machinery to carry out other essential functions. The essential role of these three proteins in yeast suggests that they are likely to have functional homologues in humans which, when identified, can aid in the development of therapeutic agents for the treatment of breast cancer.

Body

The tasks described below are derived from the two major goals of this research: (a) to further understand the mechanism of end protection in eukaryotes and (b) to extend the knowledge gained in *S. cerevisiae* to humans by identifying human homologues of the essential telomere proteins – Cdc13, Stn1 and Ten1.

Task 1. **Identify a collection of yeast homologues of the end protection complex.**
Completed and discussed in detail in 2003 Annual Report. In short, homologues of each protein (Cdc13, Stn1 and Ten1) were identified in various budding yeast species.

Task 2. **Identify potential human homologues of the end protection complex.**

a. Align sequences and use conserved regions to search for human homologues of Stn1 and Ten1 in the database

Completed and discussed in detail in 2004 Annual Report. Briefly, Hidden Markov Models were derived based on the sequence alignments derived in Task1. These models were used in database searches for putative human homologues. As stated previously, increasing data supports the idea that a protein known as human Pot1 may be the human homologue of Cdc13 [2, 9-11, 13, 19, 20]. Using our bioinformatic methods, no putative human Ten1 (huTen1) homologues were identified. A putative human Stn1 (huStn1) was identified and was found to interact with the known telomere binding protein, TRF1, by yeast two hybrid. Unpublished data from two other labs also suggests that this putative human Stn1 does indeed function at the telomere.

Task 3. **Investigate the mechanism by which Cdc13 regulates telomere end protection.**

a. Perform an evolutionary trace of identified Cdc13 homologues
Completed and discussed in detail in 2003 Annual Report.

b. Conduct mutational analysis of amino acid residues identified by the Cdc13 evolutionary trace
Completed and discussed in detail in 2003 Annual Report.

c. Conduct mutational analysis of highly conserved amino acid residues of Ten1 and Stn1
In order to further our understanding of how Stn1 and Ten1 mediate end protection I mutated highly conserved residues in Ten1. As discussed in the 2004 Annual Report, I obtained mutations at two specific residues that lead to lethal phenotypes. I previously reported that we could measure the ability of Ten1 to bind DNA by electrophoretic mobility shift assay. This shift was highly reproducible. However, because of the amt of protein needed to observe the shift, we could not unequivocally state that the shift was due specifically to the presence of Ten1 (although equimolar amounts of GST alone did not result in the same DNA shift). As such, we are currently working on other methods to determine the reason for lethality in these two Ten1 mutants. A Ten1 yeast two-hybrid plasmid has been constructed and I am currently testing how these mutations affect the ability of Ten1 to interact with both Stn1 and Cdc13.

Highly conserved amino acids have also been mutated in Stn1. The phenotypes of these alleles is currently being analyzed *in vivo*.

Task 4. **Determine whether the localization of Cdc13, Stn1 and Ten1 to the telomere is temporally regulated and if phosphorylation affects its telomere localization.**

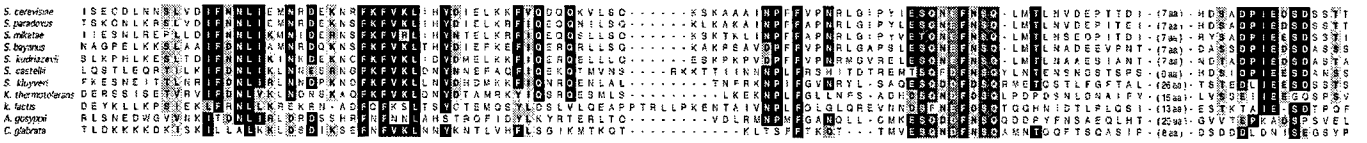
a. Perform ChIP assays on Cdc13, Stn1 and Ten1.
We have derived two new antibodies to endogenous Cdc13 – one of which recognizes Cdc13 itself, the other which recognizes a phosphospecific form of Cdc13 (see Task 4b for details). Using an epitope tagged Cdc13, the Zakian lab has previously published that Cdc13 is associated with the telomere throughout the cell cycle [17]. We are currently using the phosphospecific anitbody to see how the association of the phosphorylated Cdc13 changes across the cell cycle and also how its association changes in mutant backgrounds.

Because we do not currently have antibodies to either Stn1 or Ten1, we will be using epitope tagged versions of each protein to carry out ChIP analysis.

b. Use immunoprecipitations and Western blots to determine if the three proteins are phosphorylated in a cell cycle regulated manner.

<u>Phopshorylation of Cdc13</u>
As described in the previous report, alignment of the Cdc13 homologues I collected in Task 1 led to the identification of two conserved PI3-like kinase consensus sites (S/TQ)[8] at amino acid positions 249 and 255 (**Figure 1**). Mutations in both known yeast PI3-like kinases, Tel1 and Mec1, lead to significant defects in telomere maintenance and are therefore likely candidates for phosphorylating Cdc13 *in* vivo [3, 4, 12, 15]. These conserved motifs flank a residue central to the ability of Cdc13 to recruit telomerase to the telomere [14] so we hypothesized that Tel1-dependent phosphorylation of these two residues may regulate the ability of Cdc13 to recruit telomerase to the telomere.

Figure 1. Identification of two PI3 kinase consensus sites on Cdc13. Conserved S/Q sites are highlighted in blue. Residue mutated in the recruitment defective *cdc13-2* allele is highlighted in red.



We previously reported that a fragment of Cdc13 containing these two potential phosphorylation sites could be phosphorylated *in vitro* by a commercially available PI3-like kinase, DNA-PK, and that this event was abolished by mutating both serines to alanine. In order to determine whether this phosphorylation event occured *in vivo,* we derived a rabbit polyclonal antibody specific to Cdc13 phosphorylated at serine 255 (α-S255P). Due to technical difficulties, we were unable to make an antibody that recognized both phosphorylated S249 and S255. Using the α-S255P antibody, we have been able to demonstrate that serine S255 is indeed phosphorylated *in vivo* (**Figure 2**). This signal is specific as it disappears in the presence of phosphatase treatment.

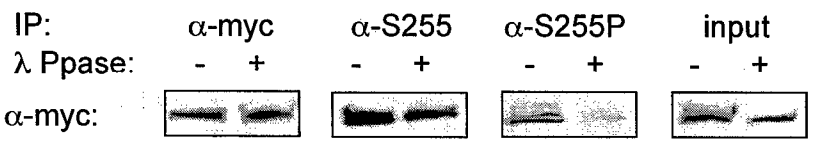


Figure 2. Cdc13-myc was immunoprecipitated using antibodies raised against an unphos-phorylated Cdc13 peptide (a-S255) or the same peptide phosphorylated at S255 (a-S255P), or with a-myc. All four westerns were probed with a-myc; extracts were untreated or incubated with l phosphatase, prior to IPs.

Connie Nugent, Ph.D., a previous postdoctoral fellow and Department of Defense award recipient in our lab, had previously reported that Cdc13 is phosphorylated in a cell cycle dependent manner. The specific phosphorylation event Dr. Nugent reported could be observed by a change in the electrophoretic mobility of an epitope tagged Cdc13. Mutation of both serine 249 and serine 255 to alanine does not abolish the shift observed by Dr. Nugent (data not shown). As such, we conclude that Cdc13 is likely phosphorylated at multiple sites that have yet to be identified. Furthermore, we are currently testing whether the phosphorylation event at serine 255 is also cell cycle dependent and also whether it is dependent on the PI3-like kinase, Tel1.

Phosphorylation of Stn1 and Ten1

Using a FLAG tagged Stn1, I have demonstrated that Stn1 appears to be phosphorylated (**Figure 3a**). Initial data suggests that this phosphorylation event may be regulated in a cell cycle dependent manner (**Figure 3b**).

I have not yet been able to construct reagents necessary for analysis of Ten1 phosphorylation.

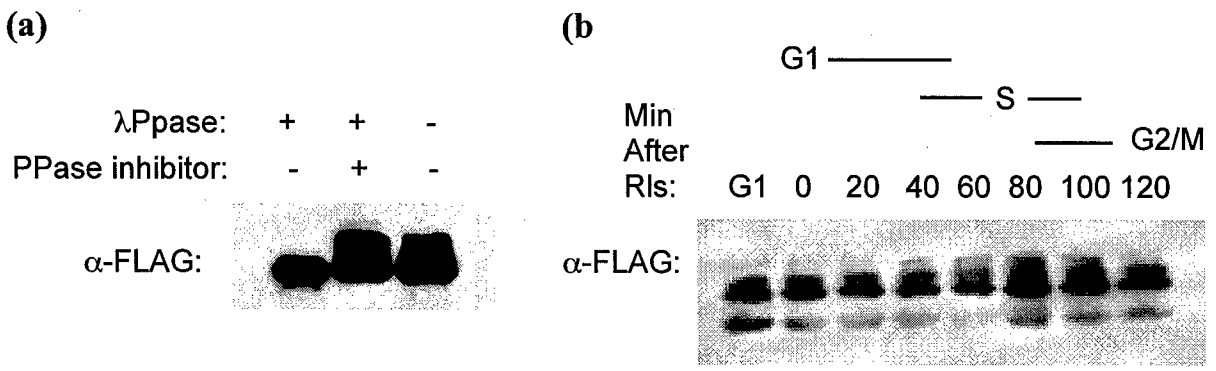


Figure 3. Stn1 is phosphorylated *in vivo* potentially in a cell cycle dependent manner. (a) FLAG tagged Stn1 was immunoprecipitated from yeast and was treated or not treated with lambda phosphatase as indicated. FLAG tagged Stn1 can not detected in crude lysate presumably due to low level expression. **(b)** Yeast strain was arrested at G1 with alpha-factor and released into the cell cycle. Phase of the cell cycle was monitored by bud index and is indicated at the top. At each timepoint, FLAG tagged Stn1 was immunoprecipitated. All samples were simultaneously run on an SDS-PAGE and western blotted with an anti-FLAG antibody.

c. Identify phosphorylated residues by mass spectroscopy.

As stated in Task 4b, we believe Cdc13 is phosphorylated at multiple sites. However, over the past year, we have chosen to initially focus on the two biologically relevant phosphorylation events we have identified instead of trying to identify more sites. Identifying additional phosphorylation sites on Cdc13 as well as Stn1 and Ten1 remains an interesting task. As such, it will indeed be worked on despite the completion of this grant.

d. Mutate these phosphorylated sites in yeast and analyze strains for deficiencies in telomere maintenance.

As described in the 2004 Annual Report, mutation of the two phosphorylation sites described in Task 4b from serine to alanine (*cdc13 S249A,S255A*) leads to a severe telomere replication defect. Interstingly, these serine to alanine mutations did not affect the ability of Cdc13 to interact with the Est1 subunit of telomerase as measured by yeast two hybrid. Mutation of both serines to the phosphomimetic aspartate, however (*cdc13 S249D,S255D*), led to an increased interaction with the Est1 subunit of telomerase.

These observations have led us to propose that the phosphorylation of Cdc13 at serines 249 and 255 are not necessary for overall telomerase recruitment. However, we do believe that phosphorylation at

these sites is necessary for bringing the overall low level of telomerase to those shorter telomeres that require elongation in each cell cycle. Recent work from the Lingner lab has demonstrated that only 10% of telomeres are elongated in each cell cycle and that short telomeres are preferentially elongated[18]. We believe that the phosphorylation event we describe is the mechanism by which short telomeres are preferentially elongated.

Note
This past year, my mentor, Dr. Vicki Lundblad, relocated from Baylor College of Medicine (Houston, TX), to the Salk Institute for Biological Sciences (La Jolla, CA).

Key Research Accomplishments
Demonstrated that Cdc13 is phosphorylated at two PI3-like kinase consensus sites *in vivo.*
Phosphorylation of Stn1 MAY be regulated in a cell cycle dependent manner.
Cdc13, Stn1 and Ten1 may function as a telomere-specific RPA.

Reportable Outcomes
Publications:
Theobald, D.L., Cervantes, R.B., Lundblad, V., and Wuttke, D.S., *Homology among telomeric end-protection proteins.* Structure, 2003. 11(9):1045-1050.

Cervantes, R.B., Post, S.M., Otero, J., Mandell, E. K. and Lundblad, V. "A telomere specific RPA mediates replication and repair at chromosome ends" *In preparation.*

Presentations:
Cervantes, R.B., Otero, J., Mandell, E.K., Post, S., and Lundblad, V. "A telomere specific RPA mediates repliation and repair at chromosome ends. (Cold Spring Harbor Meeting on Telomeres & Telomerase, 2005)

Conclusions
Identification of homologues of Cdc13, Stn1, and Ten1 has led to the discovery of two sites on Cdc13 that are phosphorylated *in vivo.* This phosphoryation event is likely to be important in directing the overall low level of telomerase to critically short telomeres. A likely next step is to identify a similar mechanism of telomerase regulation in human cells. While there are no obvious potential sites on the human homologue of Cdc13, human Pot1, sites could potentially be located on other telomeric proteins that are important in regulating telomerase recuritment. Drugs that can inhibit or otherwise regulate such an event would be invaluable in cancer treatment. Characterization on a putative human homologue of the essential telomere protein, Stn1, also continues.

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